CONTRACT NO. A933-078
FINAL REPORT
FEBRUARY 1992

Effects of Acidic Mixtures on Pulmonary Macrophage Functions:

A Pilot Study

CALIFORNIA ENVIRONMENTAL PROTECTION AGENCY

AIR RESOURCES BOARD
Research Division

EFFECTS OF ACIDIC MIXTURES ON PULMONARY MACROPHAGE FUNCTIONS: A PILOT STUDY

Final Report
Contract No. A933-078

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February 1992

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ABSTRACT

Although sulfuric acid tends to dominate the atmospheric acidity in the eastern United States, nitric acid is more prevalent in southern California. In relation to sulfuric acid, little is known regarding the pulmonary toxicity of nitric acid, especially in environmentally relevant mixtures (such as with ozone). This study had two objectives: to examine the effects of inhaled nitric acid and ozone on lung macrophage cell function, and to evaluate new endpoints with respect to their applicability to future acid inhalation studies.

The study was conducted in three parts: biological endpoint protocol development; a series of single, acute 4 hour inhalation exposures; and a series of 4 day repeated 4 hour inhalation exposures at 1/4 the acute exposure concentrations. The total exposure (concentrations of nitric acid and ozone x time) that the animals received was the same in the 4 hour and the 4 day experiments.

Certified-viral-free Fischer 344 rats were exposed, nose-only; the rats were then sacrificed, and lung lavage was performed. Lung lavage cells and lavage fluid were evaluated using a variety of endpoints related to alveolar macrophage function. Alveolar macrophage-related endpoints were the focus of this study because alveolar macrophages are critically important cells with respect to defense against infection, sequestration of foreign material, hypersensitivity reactions, lung inflammation, and

tissue destruction, all of which are known to be relevant to human health. The endpoints that were performed fell into six categories: 1) lavage cell number and type; 2) intracellular pH of lung lavage cells and lavage fluid pH; 3) lavage cell metabolism of the carcinogen precursor, benzo [a] pyrene; 4) respiratory burst and phagocytic activity; 5) in vitro production of arachidonic acid metabolites (leukotriene B_4 and leukotriene C_4) by lung lavage cells; and 6) elastolytic activity and elastase inhibitory capacity of lavage fluid and lung lavage cells.

This study found effects of inhaled nitric acid on some of the above lung macrophage parameters, although the levels of acid used were greatly elevated over the ambient concentrations. A single 4 hour exposure to 1 mg/m^3 nitric acid resulted in increased production of leukotriene B_4 by lung lavage cells in vitro, and an increase in the elastase inhibitory capacity of lung lavage fluid. Exposure to 0.25 mg/m^3 nitric acid 4 hours per day for 4 days resulted in decreased respiratory burst activity of lung lavage cells and, as in the single 4 hour exposure, an increase in the lavage fluid elastase inhibitory capacity.

Exposure to 0.6 ppm ozone for 4 hours had significant effects on a number of parameters such as lavage cell type, lavage fluid protein content, and elastase inhibitory capacity. However, for almost all endpoints examined, the interaction of nitric acid and ozone was not additive, in fact, a weak antagonistic interaction was observed.

ACKNOWLEDGEMENTS

The investigators thank Mr. Richard Mannix, Mr. Ainsworth Thomas, and Ms. Linde Nansen for their expert technical assistance during, the project, Ms. Marie Tonini for word processing and Dr. Shankar Prasad for his advice regarding the experimental design.

This report is submitted in fulfillment of contract A933-078, "Acid Mixture Toxicology," by the University of California under the partial sponsorship of the California Air Resources Board. Work was completed as of 2/28/91. This report was prepared by Dr. Christine Nadziejko and Dr. Robert Phalen.

DISCLAIMER

The statements and conclusions in this report are those of the contractor and not necessarily those of the California Air Resources Board. The mention of commercial products, their source, or their use in connection with material reported herein is not to be construed as either an actual or implied endorsement of such products.

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Figure 5b. Effect of exposure to nitric acid vapor, alone and in combination with ozone, on lung lavage fluid elastase-like activity and elastase inhibitory capacity.

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CONCLUSIONS

1) The endpoints that were found to be affected by in vivo exposure to nitric acid vapor, alone or in combination with ozone, included macrophage respiratory burst activity, production of arachidonic acid metabolites by macrophages, and lavage fluid elastase inhibitory capacity.

2) Xenobiotic metabolism by lung macrophages and elastolytic activity of lung macrophages were below the level of detection in all samples examined.

3) The intracellular pH of lung macrophages, lavage fluid pH and lavage fluid elastase activity were measurable but were not affected by in vivo exposure to acidic atmospheres.

4) The effects of nitric acid and ozone on the end points examined in this study were not additive. In fact, in most cases the effect of nitric acid and ozone in combination was less than that of either ozone alone or nitric acid alone.

5) Rats exposed to 1mg/m^3 HNO_3 for 4 hours and rats exposed to 0.25 mg/m^3, HNO_3 4 hrs/day for 4 days had comparable increases in lavage fluid inhibitory capacity (relative to air exposed controls) suggesting that this endpoint reflects cumulative effects of nitric acid exposure.

RECOMMENDATIONS

Two major recommendations can be made as a result of this research.

1) We recommend that pulmonary macrophage respiratory burst activity, production of arachidonic acid metabolites (leukotriene B_4 and leukotriene C_4) by macrophages, and lavage fluid elastase inhibitory capacity by considered for future ARB-sponsored studies of the chronic and subchronic effects of in vivo exposure to nitric acid vapor. Changes in these three endpoints, i.e. macrophage respiratory burst activity, arachidonic acid metabolism, and lavage fluid elastase inhibitory capacity, have implications with respect to the development of lung infection, asthma, and emphysema, respectively.

2) Our endpoints focussed on functions associated with the deep lung. However, nitric acid is expected to have effects on the upper airways due to its significant water solubility. Therefore, we recommend that the future studies also include investigation of effects on upper airway endpoints such as mucus production and bronchial inflammation.

INTRODUCTION

It is currently difficult, if not impossible, for regulators to establish air contamination criteria for acids. Efforts to analyze data from epidemiological studies have suggested that there is a link between acidic atmospheres and adverse health effects, but the effects of acid can't be clearly distinguished from effects produced by other co-pollutants. While sulfates and sulfuric acid contribute significantly to atmospheric acidity in the eastern United States, nitric acid is a dominant form of acid in California. The predominance of nitric acid as the major contributor to acidity in California's atmosphere compounds the difficulty of setting State standards for acid air pollution because almost all controlled exposure studies have examined the effects of acidic sulfates and very little is known about the health effects of nitric acid exposure.

One viewpoint is that the concentration of total available acidity in the air times exposure time is the determining factor in the effect of acidic atmospheres on the lung. However, it is not clear to what extent the chemical nature (nitric vs. sulfuric) and the physical state (gas vs. particles) may also play a role in the toxicity of acid air pollution. In addition, it is possible that nitric acid in ambient concentrations may significantly influence the toxicity of coexisting pollutants such as ozone.

Alveolar macrophage function was selected as the focus of this study of the health effects of nitric acid. Alveolar macrophages are critically important cells with respect

to defense against infection, sequestration of foreign material, hypersensitivity reactions, lung inflammation and tissue destruction (Fels and Cohn, 1986). These functions, if disrupted or unchecked, have implications with respect to the development of lung disease.

In this pilot study we elected to examine the following macrophage-related functions; 1) macrophage respiratory burst activity, 2) metabolism of xenobiotics by macrophages, 3) production of arachidonic metabolites by macrophages, and 4) elastolytic activity of lung macrophages and elastase inhibitory capacity of lung lavage fluid. These macrophage-related functions have implications with respect to the development of a) lung infection, b) carcinogenesis, c) asthma, and d) emphysema. In addition, the effect of in vivo exposure to acid mixtures on the intracellular pH of lung lavage cells was also determined.

This study was conducted in three phases. In the first phase, baseline studies were performed on several assays and measurements using alveolar macrophages from clean-air-exposed and ozone-exposed rats to establish standard protocols and to optimize the research evaluation methods. In the second phase, the effects of a single 4 hour exposure of rats to nitric acid, ozone, and ozone plus nitric acid on various parameters relevant to alveolar macrophage function and morphology were examined. In the third phase, the parameters that showed a response to a single 4 hour exposure were selected for additional study in rats that were exposed for 4

hours/day for 4 days. The atmospheric components used in this 4 day exposure were 1/4 the concentration of the phase 2 atmospheres. Thus, the concentration times time relationship was kept constant in the two exposures.

It should be noted that the concentrations of nitric acid vapor that were used in these acute exposure studies (1 mg/m^3 and 0.25 mg/m^3) are much higher than the levels encountered in the South Coast Air Basin. In Pasadena, HNO_3 concentrations ranged from 0.001 to 0.056 mg/m^3 with an average of .018 mg/m^3 from December 1985 through March 1986 (Munger et al., 1990).

OBJECTIVES

1. To test the hypothesis that exposure of animals to nitric acid, alone or in combination with ozone, will affect one or more parameters related to alveolar macrophage function.

2. To evaluate new endpoints for their utility in examining the effects of inhaled acidic atmospheres.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats were used for the first phase of the project and Fischer 344 rats

(230-260 gm) were used for the second and third phases of the project. The Fischer 344 rats are genetically defined and thus are expected to be less variable in their responses to pollutants than the outbred Sprague-Dawley animals.

The animals used in this study were certified viral-free male rats (Harlan Sprague Dawley, Inc., Indianapolis, IN). Rats weighing approximately 250g were delivered in filtered shipping boxes to minimize prior exposure of the animals to particulate pollutants. The rats were housed in a particle and gas-filtering laminar-flow caging system for about 1 week prior to the start of the experiment. Serologic data provided by Harlan Sprague Dawley indicated that the rats were free of respiratory viral disease. This was confirmed through serologic testing and quality control histopathologic examinations performed by our laboratory on rats from each batch of animals received. In addition, the certified viral free Fisher rats used in the second and third phases of the project were functionally evaluated for lung health. We required that greater than 95% of the sham-rats' lavaged cells be macrophages. All groups used passed this test for freedom from significant inflammation.

Exposure Methods

Groups of rats were exposed to either purified air or pollutant atmospheres using 1-m^3 stainless-steel University of Rochester-type chambers which we modified for nose-only exposure. Rats from each supply batch were randomly assigned to exposure groups and were exposed nose-only following identical experimental protocols. The chambers

were supplied with air that had passed through a coarse particulate filter, a gas (and vapor) scrubber, a humidifier and a HEPA filter. When appropriate, O_3 and/or HNO_3 were injected into the airstream just prior to the exposure chamber. The temperature and relative humidity were regulated at about 23°C and 60%, respectively.

Ozone was generated by passing medical-grade oxygen through corona-discharge ozonizers (Sander Type III, Osterberg, FRG) and then diluting the ozone with purified air. Ozone was sampled from the rat breathing zone through fluorocarbon tubing using calibrated ultraviolet light absorption continuous monitors (Dasibi Environmental Corp., Glendale, CA). An in-house ozone transfer standard was calibrated by the ARB prior to the start of the study, and the ozone monitors were subsequently calibrated at weekly intervals during the study. Vapor-phase HNO_3 was generated by atomizing a dilute, aqueous nitric acid solution using a Collison nebulizer. The droplets were then evaporated by dilution with dry, purified air, and the resulting vapor was then injected into the chamber airstream. Sampling was performed from the rat breathing zone using two filters in tandem: a teflon-coated glass fiber prefilter, which was used to check for the presence of aerosol-phase HNO_3, should any be present; and a nylon backup filter, which was used to collect HNO_3 vapor. Filter samples were extracted in aqueous media and aliquots of each extract were analyzed by ion chromatography to determine the nitrate concentration. Data obtained in this manner were then used to determine the HNO_3 concentration in the exposure chamber air.

Lung Lavage

Rats were deeply anesthetized by intraperitoneal administration of sodium pentobarbital (65mg/kg). Invasive procedures were performed only after each rat had reached a deep level of anesthesia, as determined by the loss of deep-tendon reflexes. Approximately 2-3 ml of blood was sampled from the portal vein and the aorta was severed. The trachea was exposed, cannulated with a thin-walled plastic catheter, and the diaphragm was exposed and cut away from the anterior rib cage. Rats were lavaged simultaneously in groups of 5 using a multi-channel peristaltic pump to minimize variability of the lavage procedure. The lungs of each rat were lavaged with approximately 10 ml of Hank's balanced salt solution, repeated 5 times for a total lavage volume of 50 ml. The first aliquot of lung lavage fluid was collected separately and the acellular supernatant was used for a number of assays, as described below. The cells from all five lavages were pooled and resuspended at a concentration of approximately $1x10^6$ cells per ml. Cell counts were performed using a bright line hemocytometer. An aliquot of the lung lavage cells from each rat was pelleted onto a glass microscope slide using a cytospin (Shandon, Inc., Pittsburgh, PA) and stained with Diff-Quick (Baxter Healthcare McGaw Park, IL) to determine the lavage cell differential count.

Measurement of Respiratory Burst Activity and Phagocytosis

The term "respiratory burst" refers to the ability of phagocytes to generate superoxide. Respiratory burst activity of lung lavage cells was measured by lucigenin

chemiluminescence (Williams and Cole, 1981a) after the cells were maintained in culture overnight using the following protocol.

Lung lavage cells ($0.5x10^6$) were aliquoted into sterile polystyrene cuvettes and incubated in RPMI 1640 medium (Gibco, Grand Island, NY) at 37° overnight. The culture medium was removed and the cells were preincubated in 0.4ml Dulbecco's phosphate buffered saline plus 5 mM glucose for 30 minutes at 37°C. Respiratory burst activity was stimulated by adding three different agents which activate the cell via three different pathways: 1) opsonized zymosan (50μl of 2.5 mg/ml); 2) phorbol myristate acetate (PMA) (24μl of 1μg/ml); and 3) formyl methionyl-leucyl-phenylalanine (20μl of 1μg/ml). Lucigenin was added (50μl of 2mM) and light emission (produced by the reaction of lucigenin with superoxide) was measured using a LKB 1251 luminometer (Pharmacia-LKB Nuclear Division, Gaithersburg, MD). Light emission was monitored until the peak light emission rate was reached and light emission began to decline. Data was expressed as peak height (in mV) per mg adherent cell protein.

Respiratory burst activity was also measured in freshly isolated lung lavage cells using a cytochrome c reduction technique (Kemmerich et al., 1987). Freshly-isolated lung lavage cells spontaneously produce superoxide for several hours in the absence of any stimulating agents (Williams and Cole, 1981b). For technical reasons, this spontaneous respiratory burst activity is difficult to measure reproducibly using

lucigenin chemiluminescence; however, we found that detection of superoxide production by cytochrome c reduction was a suitable method for measuring respiratory burst activity in freshly isolated cells. Lung lavage cells ($0.2x10^6$) were added to microtiter plate wells and cytochrome c (100μm) was added with and without respiratory burst stimulants described below. Cytochrome c reduction (OD at 550 nm - OD at 540 nm) was measured after incubation at 37° for 30 minutes. Spontaneous respiratory burst activity was measured, as well as opsonized zymosan-stimulated and PMA-stimulated activity. Superoxide dismutase (SOD) (60 units) was added to duplicate samples. Data was expressed as SOD inhibitable cytochrome c reduction in n mols/30 minutes/mg adherent cell protein.

Phagocytosis was measured by adding $1x10^5$ lung lavage cells in 0.6 ml HEPES (N-2-hydroxyethylpiperazine - N-2-ethane sulfonic acid) buffered Hank's balanced salt solution in wells of a 8 chamber culture slide. Non-adherent cells were removed after 45 minutes and 0.5 ml HEPES buffered salt solution containing 0.1 ml of a 009% w/v solution of fluorescent latex beads, 1 micron in diameter, was added. After 90 minutes incubation, the cells were rinsed, the slide was air dried and the extracellular beads were dissolved by dipping the slide in methylene chloride for 15 seconds (Burleson et al., 1987). The cells were stained with Diff-Quick. Cells containing one or more intracellular beads were identified by fluorescence microscopy. Results were expressed as the % of the total macrophages containing intracellular beads.

Xenobiotic Metabolism

Aryl hydrocarbon hydroxylase activity was measured in lung lavage cells by measuring the conversion of benzo [a] pyrene (BP) to monohydroxyl - BP, a fluorescent compound (Dehnen et al., 1972). Lung lavage cells were allowed to attach to plastic culture dishes ($2x10^6$ cells/rat). The adherent cells were scraped from the plate and stored at -70°C until analysis. The cell pellet was suspended in 50mM Tris pH 7.6 followed by the addition of 1.25 μ mol NADPH (nicotinamide adenine dinucleotide phosphate, reduced form) and 0.25 μ mol benzo [a] pyrene in a final volume of 2.5 ml. After 30 minutes of incubation at 37°C, 0.5ml of 10% triton x 100 (w/v) in triethylamine was added followed by .025 ml of 5% EDTA. Fluorescence was measured at 434 nm excitation and 525 nm emission, using 3-hydroxy benzo [a] pyrene as a standard. Liver microsome samples were used as positive controls.

Arachidonic Acid Metabolism

In vitro production by lung lavage cells of two arachidonic acid metabolites, leukotriene B_4 (LTB_4) and leukotriene C_4 (LTC_4), was measured by ELISA (enzyme linked immunosorbant assay). Lung lavage cells were suspended in RPMI 1640 medium at a concentration of $1x10^6$ cells/ml and incubated in 24 well culture dishes at 37°C. Non-adherent cells were removed after 45 minutes by removing the culture medium, rapidly centrifuging it, and adding the cell-free supernatant back to the adherent cells. The lung lavage cells were cultured for a additional 3 1/4 hours, the medium was removed, centrifuged and the cell free supernatant was stored at -70°C

until analysis. LTB_4 and LTC_4 levels in the conditioned culture medium was measured using an ELISA kit purchased from Cayman Chemical Co (Ann Arbor, MI) using the manufacturer's protocol.

Elastase Activity and Elastase Inhibitory Capacity

Elastase activity in the first aliquot of lung lavage fluid was measured by incubating 100μl of lung lavage fluid with 100μl of 2mM succinyl, alanine, alanine p nitroanilide (SLAPN) at pH 7.8 for 18 hours at 37°C. Absorbance at 410 nm was determined using a microtiter plate reader. Pancreatic elastase was used as a calibration standard. In vitro elastolytic activity of cultured lung lavage cells was determined by co-culturing $1x10^6$ lung lavage cells in gentamicin-supplemented RPMI 1640 medium in tissue culture dishes coated with fluorescein-labeled elastin (Senior et al., 1989). Release of soluble elastin into the medium was measured at 24 hours and 72 hours of culture by centrifuging the culture medium, adding 0.1M Tris (pH 9) to the supernatant, and measuring fluorescence at 495 nm excitation, 520nm emission. Pancreatic elastase was used as a positive control. Elastase inhibitory capacity of the first aliquot of lavage fluid aliquot was measured by incubating 300μl lung lavage fluid with various amounts of pancreatic elastase (0.25 - 1.0 μg, 130 U/mg), at pH 8.8 for 5 minutes at room temperature followed by addition of 2 mg fluorescein-labeled elastin (Pickrell et al., 1987). The assay mixture was incubated at 37°C for 30 minutes in a roller bottle, followed by centrifugation and measurement of the fluorescence in the supernatant. The amount of elastase that was inhibited 50 ±

10% by lung lavage fluid from clean air-exposed rats was used to determine elastase inhibitory capacity of lavage fluid from rats exposed to the various atmospheres.

Intracellular and Extracellular Lung Lavage Cell pH

Lung lavage fluid was obtained within two hours of exposure. A 1 ml sample of the first lavage aliquot was stored in an air-tight syringe at 4°C and analyzed for pH and pCO_2 using a blood gas analyzer. Intracellular pH of lung lavage cells was measured by suspending lung lavage cells at a concentration of $1x10^6$/ml in the lung lavage fluid (from the same rat as the cells) and allowing the cells to adhere to a round glass coverslip (13 mm diameter). The non-adherent cells were removed and lung lavage fluid containing the fluorescent pH indicator BCECF-AM, (Molecular Probes, Eugene, OR) (the cell permeant form of BCECF) was added, at a concentration of 7 μg/ml (Lubman et al., 1989). The cells were incubated with the indicator for 45 minutes during which time the indicator diffused into the cells, the ester group was removed by intracellular esterases, and the fluorescent BCECF became trapped within the cells. The coverslip was gently rinsed, and positioned in a square fluorimeter cuvette using a hairpin to hold the coverslip at an angle of 45°. Fluorescence was determined at excitation wavelengths of 506 nm and 430 nm, emission wavelength of 526 nm. The ratio of the two readings was calculated. Buffered solutions with pH values ranging from 6.6-7.4 were then used to plot a standard curve of intracellular pH versus the ratio of fluorescence at 506 nm excitation and 430 nm excitation as follows. The medium was removed from the cuvette and replaced with a buffered solution

containing 130 mM KCL, 10 mM NaCL 1mM CaCL 1mM Mg SO_4, 2mM NaH_2 PO_4, 5mM glucose and 6mM HEPES of known pH. Nigericin (10μm) was added to equilibrate intracellular and extracellular pH, and fluorescence was determined as above.

Statistical Analysis

A batch correction factor (as described by Lee, Mustafa and Afifi 1990) was applied to all data to eliminate artifactual findings due to batch to batch variations. Day to day reproducibility was difficult to achieve in some assays. If batch effects were not corrected, statistically significant differences could have possibly resulted from batch to batch variation and not from exposure-related difference among groups. The correction factor used was Cg/Cb, where Cg is the mean value for all controls and Cb is the mean control value for a given sacrifice batch of rats. All of the endpoint data (except lavage cell type data) were multiplied by the appropriate correction factor, and the means, standard deviations and statistical significances were calculated and are shown in the figures. Statistical significance was determined by the Kruskel-Walles method. When a statistically significant p value ($p < .05$) was obtained by the Kruskel-Walles test, the pair-wise Mann-Whitney U test (for multiple comparisons) was used to determine which results were significantly different.

RESULTS

Atmosphere Characteristics

As was previously mentioned, three distinct exposure protocols were conducted. The first protocol involved a single 4 hour exposure to a) purified air and b) 0.6 ppm O_3. Animals from this exposure protocol were used to obtain baseline data on the various assays, both in control animals and in animals expected to have some lung injury. No technical problems were encountered in this initial phase of the study. The second protocol involved single 4 hour exposures to the following atmospheres: (a) purified air, (b) 0.6 ppm O_3, (c) 1.0 mg/m^3 HNO_3 and (d) 0.6 ppm O_3 plus 1.0 mg/m^3 HNO_3. The third protocol involved 4 consecutive days of exposure (4 hours per day) to the following atmospheres: (a) purified air, (b) 0.15 ppm O_3, (c) 0.25 mg/m^3 HNO_3 and (d) 0.15 ppm O_3 plus 0.25 mg/m^3 HNO_3. Summaries of the atmosphere characteristics for both studies are included in Tables A and B. The mean values for temperature, humidity, nitric acid concentration, and ozone concentration of the atmospheres were within 10% of the target values. No aerosol-phase HNO_3 was detected indicating that essentially all of the HNO_3 was in the vapor phase.

Lavage Cell Number and Type

Clean Air, 1mg/m^3 HNO_3, 0.6 ppm O_3, 1mg/m^3 HNO_3 plus 0.6 ppm O_3; 4 hr/day for 1 day. Figures 1a and 1b show the effects of the various atmospheres on the lavage cell population. A single 4 hr exposure to 0.6 ppm O_3 resulted in an increased

TABLE A
SUMMARY OF ATMOSPHERE CHARACTERISTICS
ACID MIXTURE TOXICOLOGY STUDY (SINGLE FOUR HOUR EXPOSURES)

Exposure Atmosphere	Parameter/ Pollutant	Target	Measured (Mean ± SD n)
Purified Air	Relative Humidity	60%	60.7 ± 1.0 108
	Temperature	22-24°C	23.7 ± 0.8 108
0.6 ppm O_3	Relative Humidity	60%	60.7 ± 1.0 108
	Temperature	22-24°C	23.7 ± 0.8 108
	Ozone	0.6 ppm	0.61 ± 0.02 108
1.0 mg/m^3 HNO_3	Relative Humidity	60%	60.9 ± 0.9 55
	Temperature	22-24°C	23.3 ± 0.5 55
	Nitric Acid	1.0 mg/m^3	1.08 ± 0.13 12*
0.6 ppm O_3 + 1.0 mg/m^3 HNO_3	Relative Humidity	60%	60.5 ± 1.0 53
	Temperature	22-24°C	24.1 ± 0.8 53
	Ozone	0.6 ppm	0.60 ± 0.01 53
	Nitric Acid	1.0 mg/m^3	1.01 ± 0.12 12*

TABLE B
SUMMARY OF ATMOSPHERE CHARACTERISTICS
ACID MIXTURE TOXICOLOGY STUDY (FOUR HOURS/DAY, FOUR DAYS)

Exposure Atmosphere	Parameter/ Pollutant	Target	Measured (Mean ± SD n)
Purified Air	Relative Humidity	60%	60.2 ± 0.9 180
	Temperature	22-24°C	22.5 ± 0.8 180
0.15 ppm O_3	Relative Humidity	60%	60.2 ± 0.9 180
	Temperature	22-24°C	22.5 ± 0.8 180
	Ozone	0.15 ppm	0.15 ± 0.01 180
0.25 mg/m^3 HNO_3	Relative Humidity	60%	60.4 ± 0.9 90
	Temperature	22-24°C	22.3 ± 0.6 90
	Nitric Acid	0.25 mg/m^3	0.27 ± 0.08 10*
0.15 ppm O_3 + 0.25 mg/m^3 HNO_3	Relative Humidity	60%	60.0 ± 0.8 90
	Temperature	22-24°C	22.8 ± 0.8 90
	Ozone	0.15 ppm	0.15 ± 0.01 90
	Nitric Acid	0.25 mg/m^3	0.26 ± 0.09 10*

* Data are means of daily average values.

Note: No aerosol-phase HNO_3 was observed; all of the HNO_3 present was in the vapor phase.

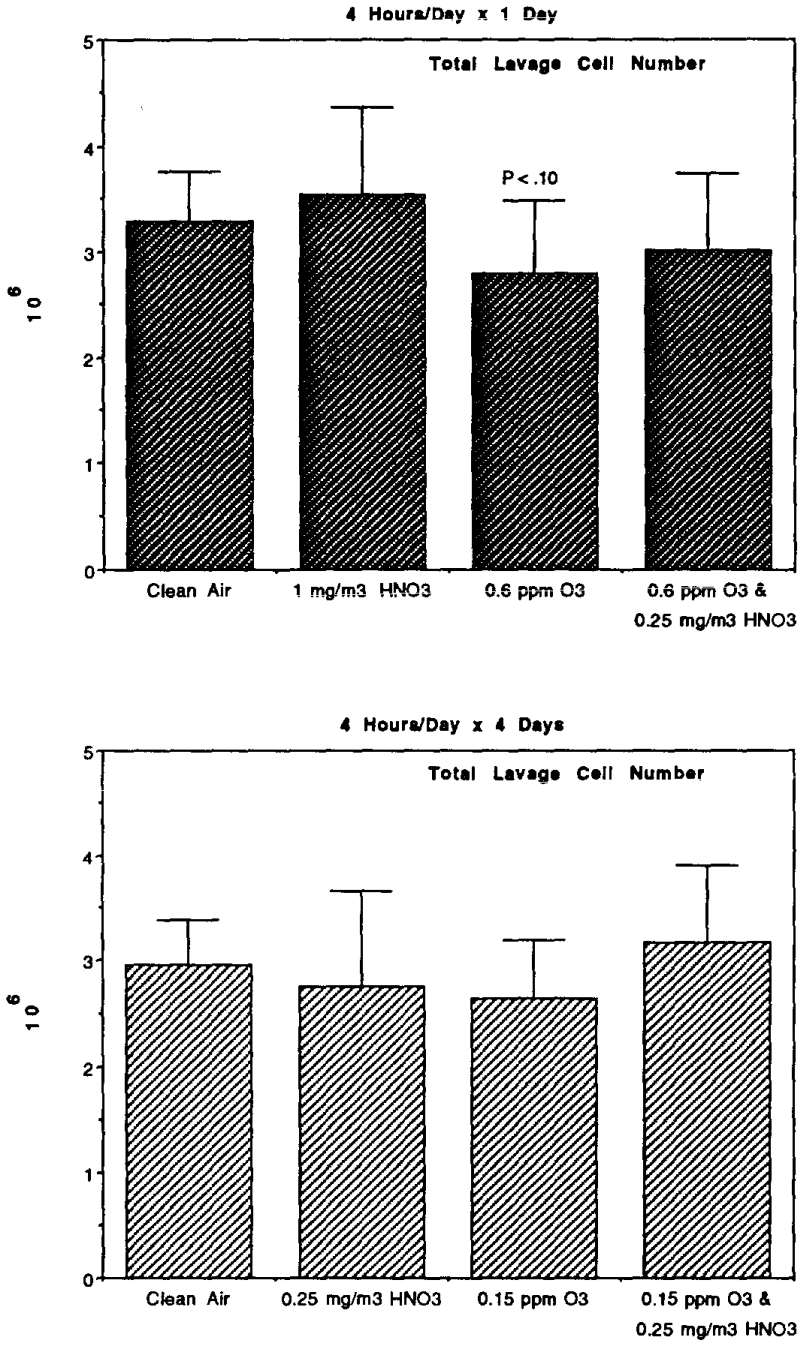
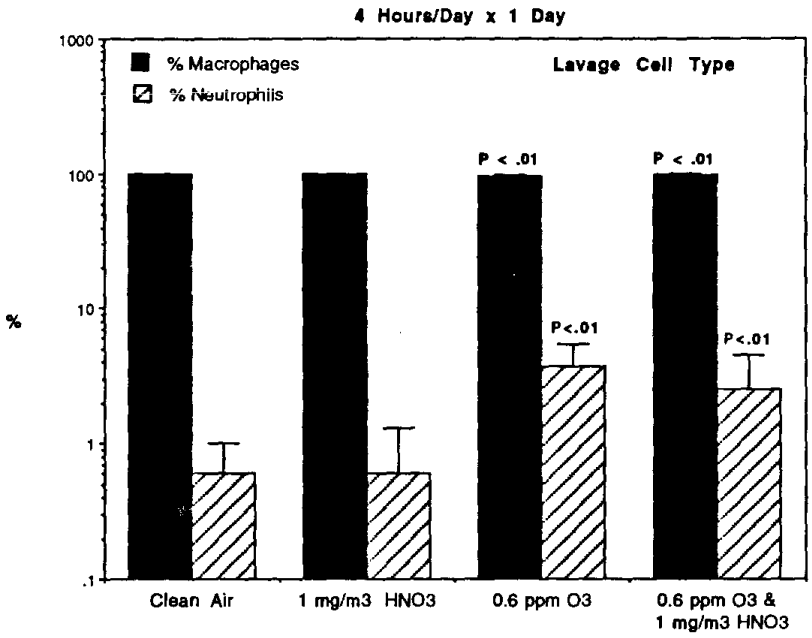


Figure 1a. Effect on in vivo exposure to nitric acid vapor, ozone, and ozone plus nitric acid on lavage cell population number. Rats were lavaged 18 hours after the final exposure. There were 20 rats in each exposure group. Average, standard deviation and statistical significance relative to the clean air group are depicted in the figures.



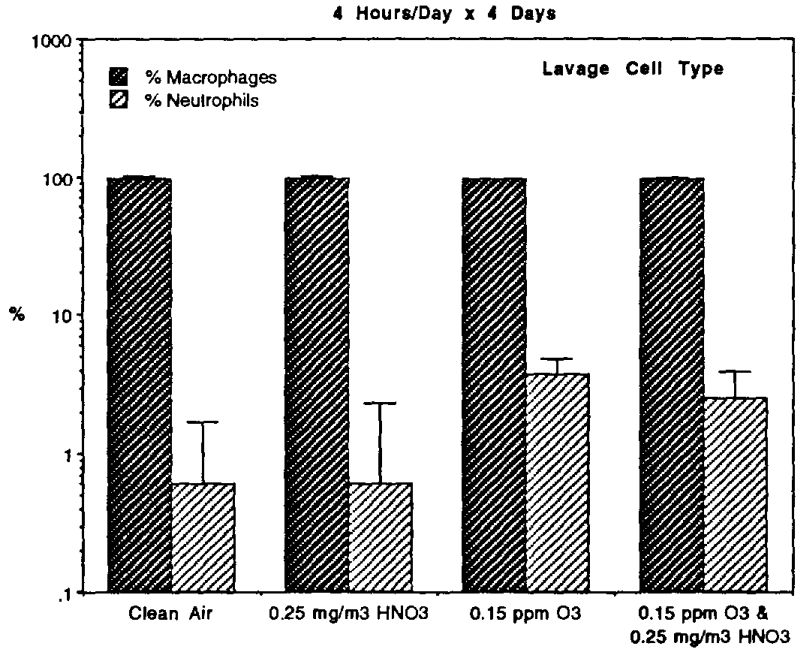


Figure 1b. Effect of in vivo exposure to nitric acid vapor, ozone and ozone plus nitric acid on lavage cell type. Rats were lavaged 18 hours after the final exposure. There were 20 rats in each exposure group. Average, standard deviation and statistical significance relative to the clean air group are depicted in the figures.

percentage of neutrophils and a corresponding decrease in macrophages in the lavage cell population (obtained 18 hrs after exposure). Nitric acid exposure alone had no effect, but the combination of HNO_3 plus O_3 also resulted in an increased percentage of neutrophils, although to a lesser degree ($p<.10$) than with O_3 alone. A slight decrease in the total number of lavage cells was observed in the animals exposed to ozone alone, but not in animals exposed to ozone plus nitric acid. There were no significant differences in other cell types (monocytes, lymphocytes and eosinophils). The number of red blood cells per high-power microscopic field was also estimated and showed no significant differences relative to the clean air group.

Clean Air, 0.25 mg/m^3 HNO_3, 0.15 ppm O_3, 0.25 mg/m^3 HNO_3 plus 0.15 ppm O_3; 4 hrs/day for 4 days. No significant differences from controls or among exposed groups seen in lavage cell number or lavage cell type.

Intracellular and Extracellular pH

Clean Air, 1 mg/m^3 HNO_3, 0.6 ppm O_3, 1 mg/m^3 HNO_3 plus 0.6 ppm O_3; 4 hr/day for 1 day. The pH of lung lavage fluid and the intracellular pH of lung lavage cells were measured to determine if exposure to the acidic atmosphere affected the pH balance of the lung and airways either directly by deposition and retention of acid, or indirectly due to injury and loss of pH regulation.

Figure 2 shows the results of intracellular pH measurements of alveolar macrophages

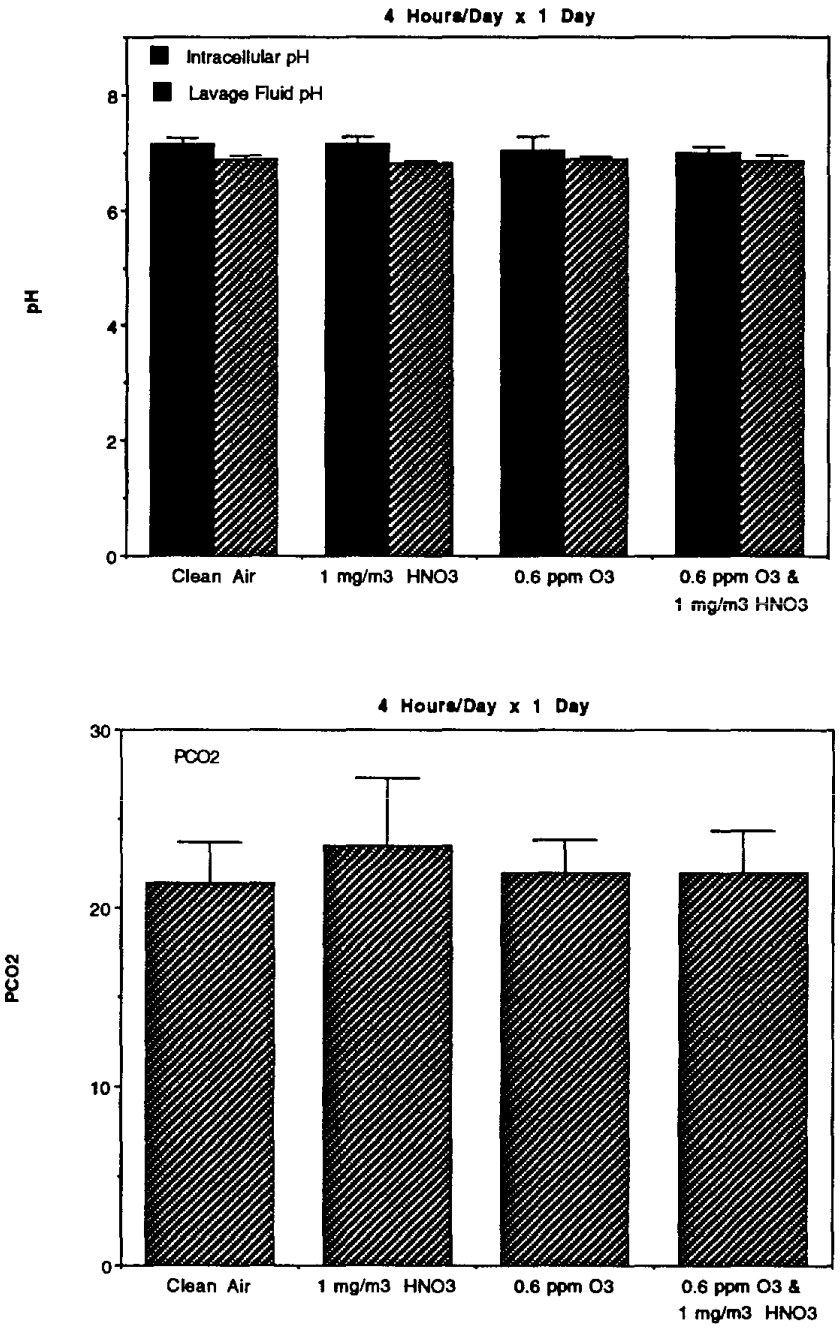


Figure 2. Effect of in vivo exposure to nitric acid vapor, ozone and ozone plus nitric acid on lavage fluid pH and intracellular pH of lung lavage cells. Rats were lavaged within two hours of exposure. There were ten rats in each exposure group. Average and standard deviation are depicted in the figures. There were no statistically significant differences ($p > .10$) relative to the clean air group.

and lavage fluid obtained within two hours of exposures. There were no significant differences among any of the groups in intracellular or lavage fluid pH. Because the Hank's balanced salt solution used to lavage the lung does have some buffering capacity, mostly in the form of H_2CO_3 (approximately 4 meq/liter), it is possible that the acidity of lavage fluid was underestimated. However, the pH of the lavage fluid that we measured (6.8-7.0) is essentially the same as the value previously reported for the alveolar lining layer (Nielson et al., 1981).

Although the lavage fluid pH averaged 6.8-6.9, when the lung macrophages were incubated in the lung lavage fluid, the intracellular pH was 7.0-7.2 on average. Recent studies have examined the mechanisms by which lung macrophages regulate intracellular pH in a relatively acidic environment, such as the lung lining layer (Bidani and Brown, 1990). It has been shown that H^+ ions are actively transported out of the lung macrophage and that this process is so efficient that the intracellular pH can increase after acidification at a rate of 0.2 pH units/minute (Bidani and Brown, 1990). In the present study, the systems involved in regulating macrophage intracellular pH were not affected by any of the exposure conditions, including 0.6 ppm O_3. Further investigation is needed to determine the sensitivity of macrophage intracellular pH regulatory mechanisms to various forms of inhaled toxicants.

Xenobiotic Metabolism

Clean Air, 1 mg/m^3 HNO_3, 0.6 ppm O_3, 1 mg/m^3 HNO_3 plus 0.6 ppm O_3; 4 hr/day for 1 day. Aryl hydrocarbon hydroxylase activity was below the level of detection in all lung macrophage samples from all exposure groups. Aryl hydrocarbon hydroxylase activity was easily detectable in liver microsomes, which were also assayed at the

same time as a positive control. It has been previously reported that exposure to ozone at concentrations of as low as 0.2 ppm for 2 weeks results in increased aryl hydrocarbon hydroxylase activity in the lung as a whole (Takahashi and Miura, 1990). It has been suggested that the increase in activity was due to a shift in the cell population to cell types which constitutively have high levels of the enzyme, rather than an overall induction of activity. Our results are in agreement with this interpretation.

Respiratory Burst Measurements

Clean Air, 1 mg/m^3 HNO_3, 0.6 ppm O_3, 1 mg/m^3 HNO_3 plus 0.6 ppm O_3; 4 hrs/day for 1 day. Respiratory burst activity was measured by lucigenin chemiluminescence on adherent lung lavage cells that were obtained 18 hours after exposure and maintained in culture for an additional 20 hours. No spontaneous respiratory burst activity was detectable under these conditions. The respiratory burst stimulants, phorbol myristic acetate and formyl methionyl-leucyl-phenylalanine, caused only minimal stimulation of respiratory burst activity (data not shown). Opsonized zymosan-stimulated respiratory burst activity was easily detectable by lucigenin chemiluminescence and was found to be significantly lower in the group exposed to both nitric acid and ozone although neither ozone alone nor nitric acid alone had any effect (Figure 3a).

Clean Air, 0.25 mg/m^3 HNO_3, 0.15 ppm O_3, 0.25 mg/m^3 HNO_3 plus 0.15 ppm O_3; 4 hrs/day for 4 days. Measurement of opsonized zymosan-stimulated respiratory burst activity by lucigenin chemiluminescence of lavage cells obtained from animals exposed to the same total dose of nitric acid and ozone as above, except at 1/4 the

concentration and 4 times the duration as above, showed that there was no effect of any of the atmospheres on respiratory burst activity, measured after the lung lavage cells had been maintained in culture for 20 hours (Figure 3a). The ability of the lung lavage cells to phagocytize latex particles was not significantly different from controls in any of the exposure groups (Figure 3b). As mentioned in the methods section, lucigenin chemiluminescence is not suitable for measuring respiratory burst activity in freshly isolated rat alveolar macrophages. However, it was possible that the macrophages recovered during the overnight culture period prior to measurement of leucigenin chemiluminescence. Therefore, we used a cytochrome c reduction assay to measure spontaneous and stimulated respiratory burst activity in lung lavage cells immediately after isolation. As shown in figure 3b significant decrease was seen in spontaneous and PMA stimulated cytochrome c reduction in lung lavage cells from rats exposed to HNO_3 alone, but not in combination. Opsonized zymosan-stimulated cytochrome c reduction was the same in all groups.

Arachidonic Acid Metabolism

Clean Air, 1 mg/m^3 HNO_3, 0.6 ppm O_3, 1 mg/m^3 HNO_3 plus 0.6 ppm O_3; 4 hrs/day for 1 day. Lung lavage cells were cultured for 4 hours after isolation and the medium was analyzed for LTB_4 and LTC_4 by ELISA. LTB_4 release was markedly greater in macrophages from HNO_3-exposed rats (Figure 4). LTC_4 levels were also increased in this group, but the difference was not statistically significant ($p>.10$). Lung lavage cells from ozone-exposed animals released significantly less LTC_4 as compared to controls. Although leukotriene B_4 is a chemotactic factor, there was no increase in neutrophils in the lavage cell population from nitric acid exposed animals, so it is as not likely that leukotriene B_4 was increased in the lavage fluid. This suggests that

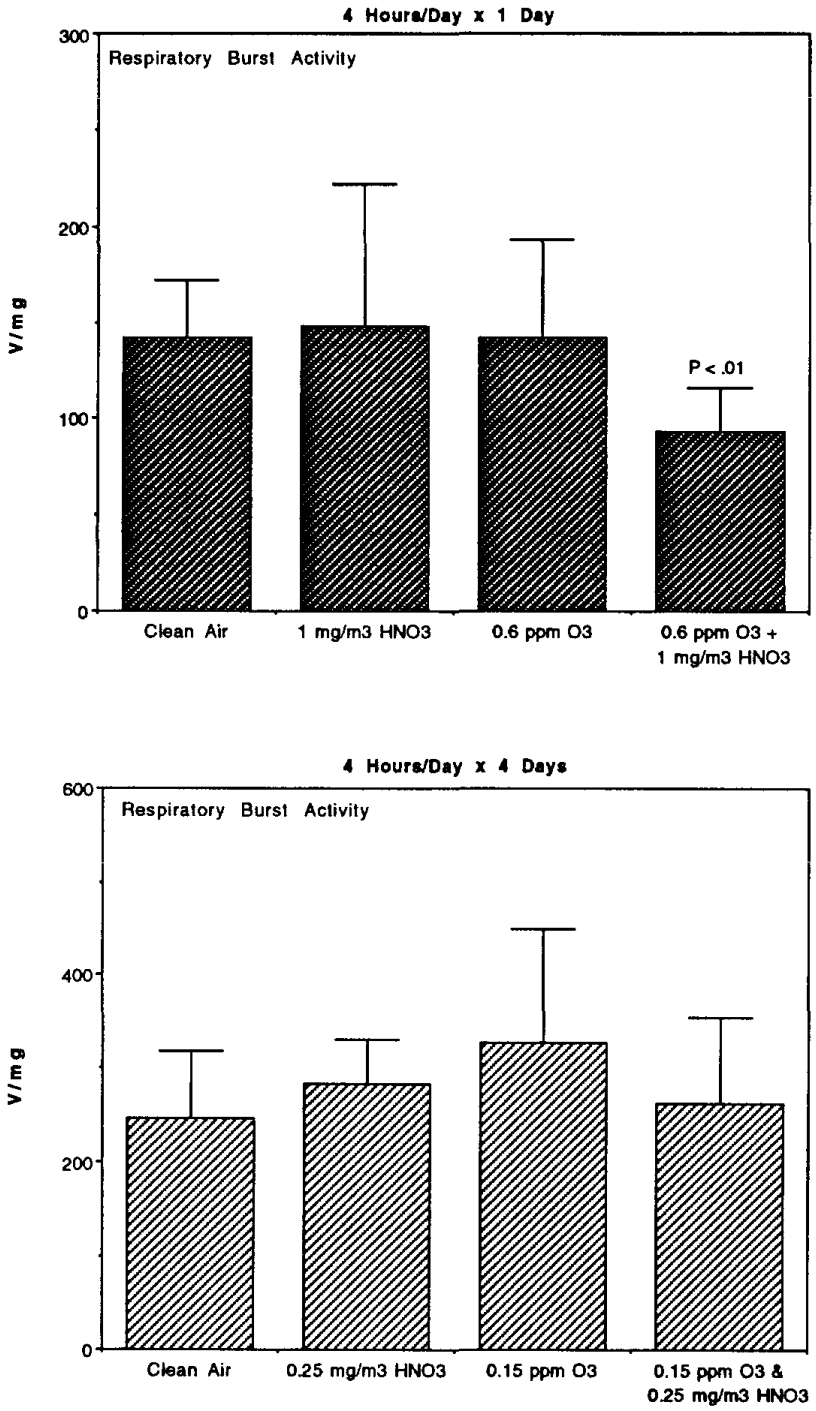


Figure 3a. Effect in vivo exposure on respiratory burst activity of lung lavage cells following short term cell culture. Ten rats per group were lavaged 18 hours after the final exposure and the lung lavage cells were maintained in cell culture for 20 hours. Respiratory burst activity was stimulated by opsonized zymosan and detected by lucigenin chemiluminescence as described in the methods. Average, standard deviation and statistical significance relative to the clean air group are shown in the figures.

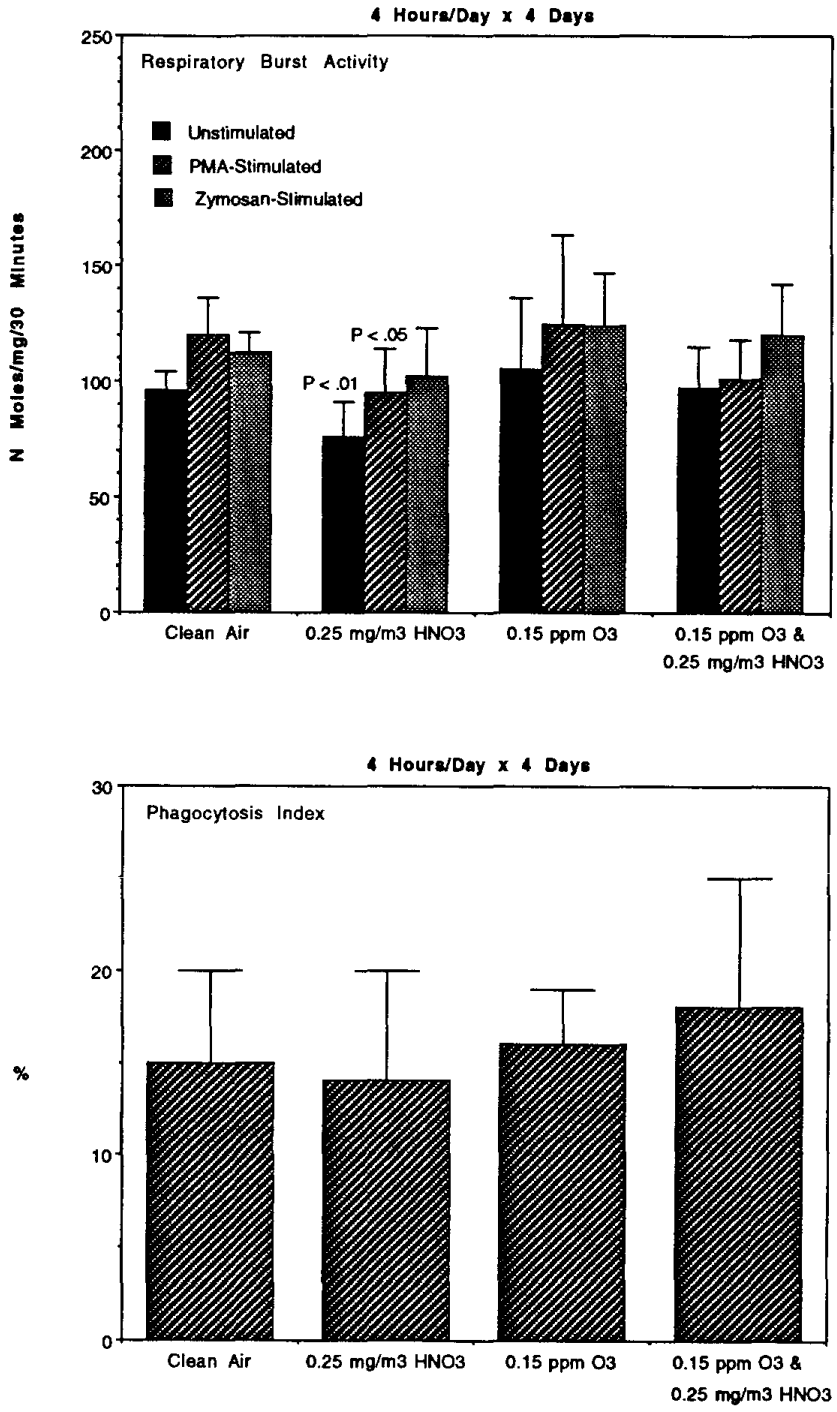


Figure 3b. Effect of <u>in</u> <u>vivo</u> exposure on respiratory burst activity and phagocytic index of freshly isolated lung lavage cells. Ten rats per group were lavaged 18 hours after the final exposure. Spontaneous and stimulated respiratory burst activity were measured by the reduction of cytochrome c as described in the methods. Average, standard deviation and statistical significance relative to the clean air group are shown in the figures.

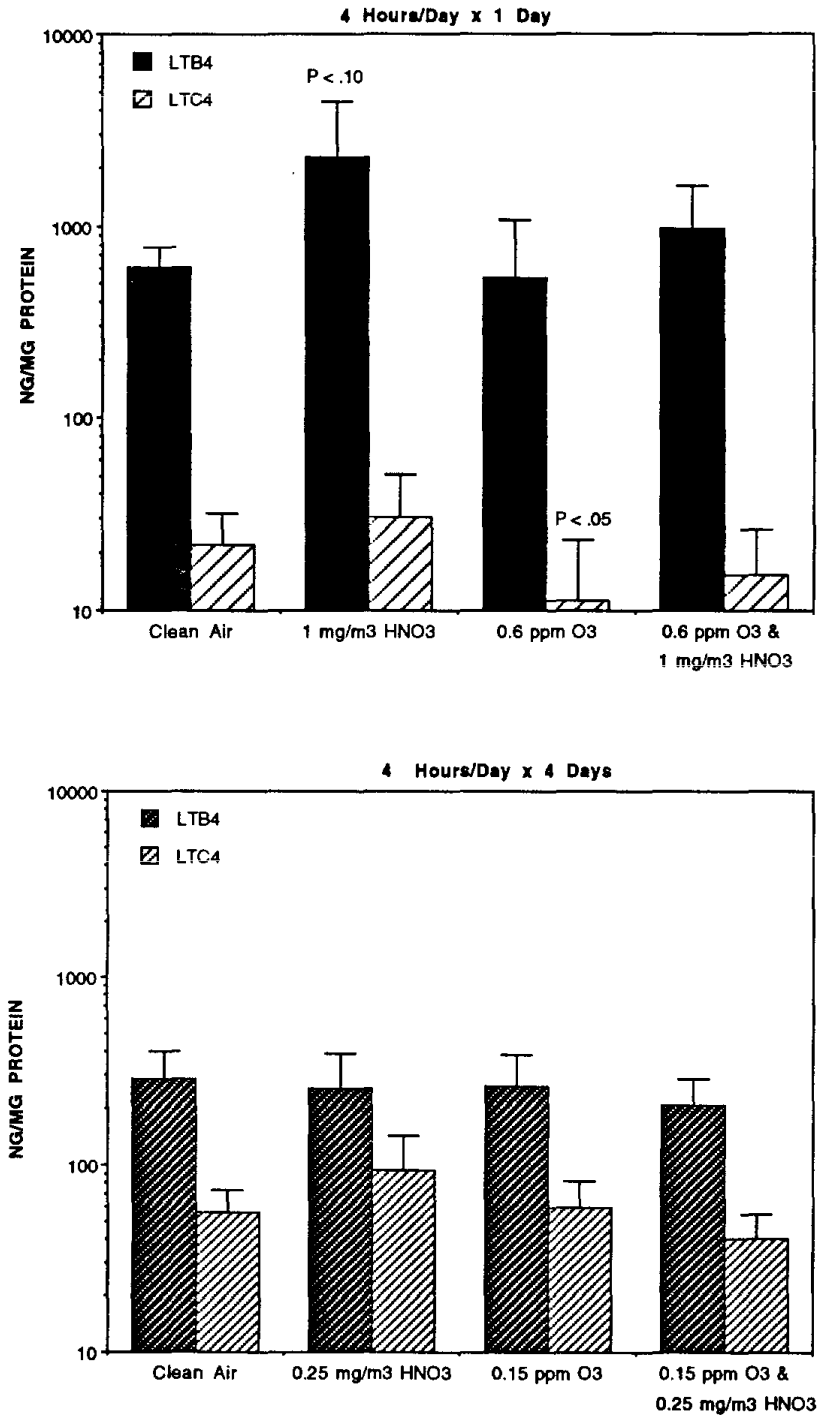


Figure 4. Effect of in vivo exposure on leukotriene production by lung lavage cells. Lung lavage cells were obtained from 10 rats per group 18 hours after the final exposure. Lavage cells were maintained in culture for 4 hours. The conditioned medium was analyzed for leukotriene B_4 (LTB_4) and leukotriene C_4 (LTC_4) levels. Average, standard deviation and statistical significance relative to the clean air group are shown in the figures.

nitric acid exposure may have "primed" the macrophages to release increased amounts of leukotriene B_4 in response to stimulation, which in this case was the contact of macrophages with the culture dish (Kouzan et al., 1988).

Clean Air, 0.25 mg/m^3 HNO_3, 0.15 ppm O_3, 0.25 mg/m^3 HNO_3 plus 0.15 ppm O_3; 4 hrs/day for 4 days. No statistically significant differences in macrophage production LTB_4 or LTC_4 were noted relative to controls.

Elastase Activity and Elastase Inhibitory Capacity

Clean Air, 1 mg/m^3 HNO_3, 0.6 ppm O_3, 1 mg/m^3 HNO_3 plus 0.6 ppm O_3; 4 hrs/day for 1 day. Lung lavage cells were cultured in serum-free medium in elastin-coated culture dishes for up to 72 hours. There was no detectable elastolytic activity by any of the lung lavage cell samples from any of the above exposure groups. Pancreatic elastase was used as a positive control. In vitro elastolytic activity by lung lavage cells has been described for human alveolar macrophages using a similar assay system. However, in previously reported research, alveolar macrophages from mice were found to require plasmin for the activation of elastase activity in vitro (Chapman and Stone, 1984).

Lung lavage fluid was also examined for protein (Figure 5a) and for elastase activity using the synthetic substrate SLAPN (succinyl, leucine, alanine, alanine, alanine, p nitroanilide). Elastase-like activity was detected in all exposure groups (Figure 5b); however, no significant differences were noted between the groups. Synthetic elastase substrates are very sensitive indicators of elastase activity. However, these substrates lack specificity (Niederman et al., 1984). Additional assays using native

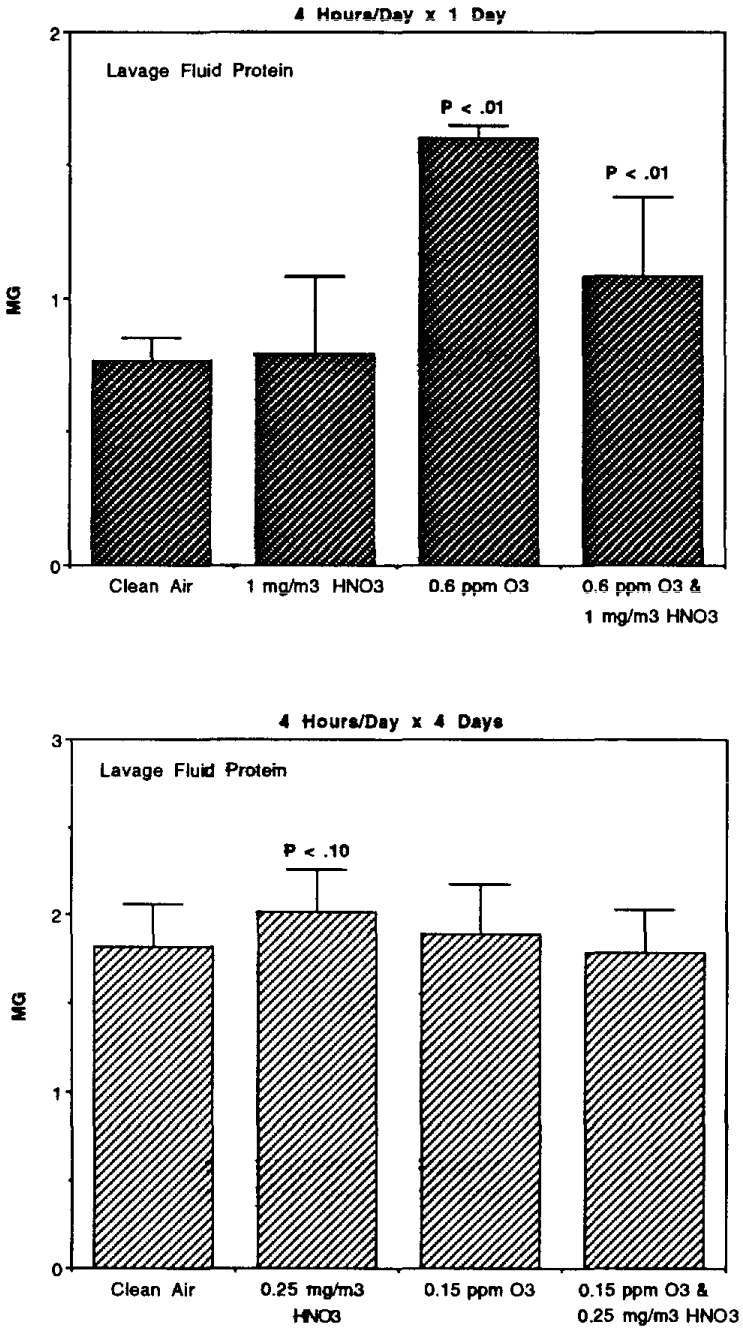
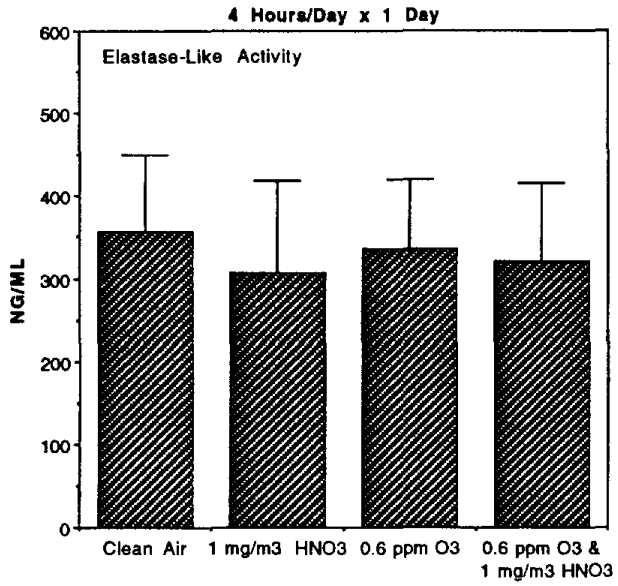
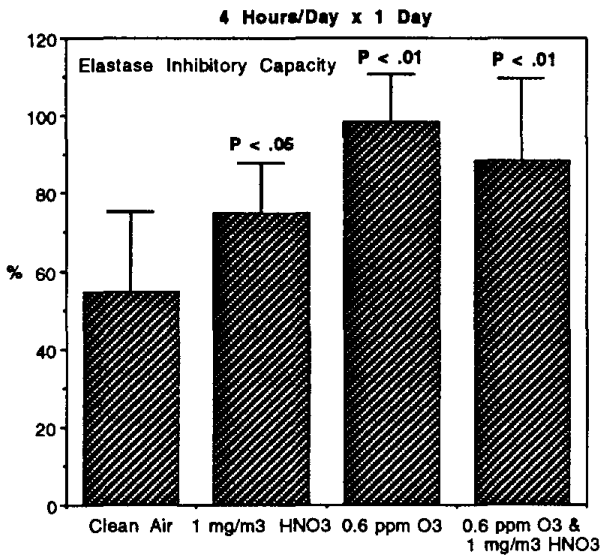
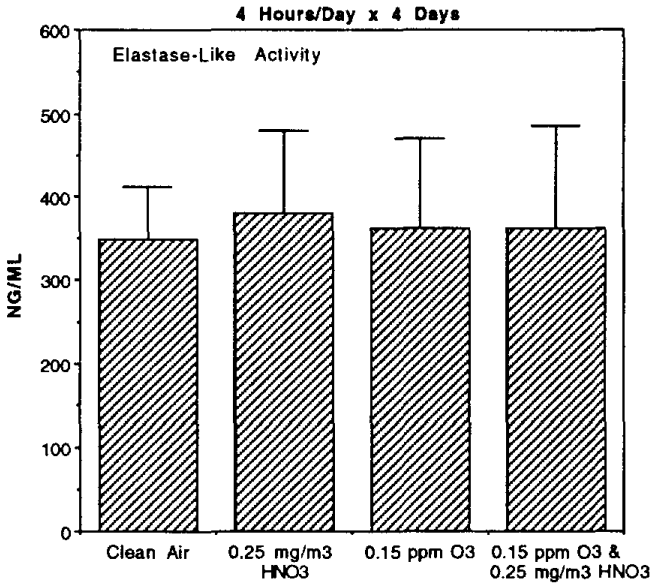
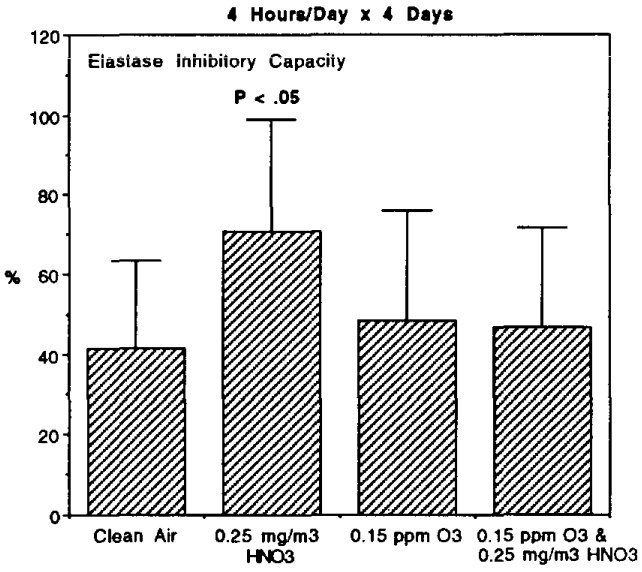


Figure 5a. Effect of exposure to nitric acid vapor, or ozone alone, and in combination on lung lavage fluid protein content. Lavage fluid was obtained from 10 rats per group 18 hours after the final exposure. Average, standard deviation and statistical significance relative to the clean air group are depicted in the figures.









Effect 5b. Effect of exposure to nitric acid vapor, or ozone alone and in combination on lung lavage fluid elastase-like activity and elastase inhibitory capacity. Lavage fluid was obtained from 10 rats per group 18 hours after the final exposure. Average, standard deviation and statistical significance relative to the clean air group are depicted in the figures.

elastin as a substrate would be necessary to determine if the elastase-like activity that was detected using SLAPN was due to elastase or some other enzyme. However, it is not likely that true-elastase activity was present because the lavage fluid had inhibitory activity toward pancreatic elastase as shown in Figure 5b. The elastase inhibitory capacity was significantly increased in groups exposed to ozone alone, nitric acid alone and ozone plus nitric acid. Lavage fluid protein content was also increased in ozone exposed animals. Lavage fluid protein was also increased relative to controls by exposure to the combination of ozone and nitric acid; however, the protein content of the lavage fluid from this group was significantly less than that of the group exposed to ozone alone (Figure 5a).

Clean Air, 0.25 mg/m^3 HNO_3, 0.15 ppm O_3, 0.25 mg/m^3 HNO_3 plus 0.15 ppm O_3; 4 hrs/day for 4 days. In vitro elastolytic activity of lung lavage cells was not examined, given the uniformly negative results described above. Lavage fluid elastase-like activity was essentially the same in all exposure groups (Figure 5b). The lung lavage protein content was slightly elevated in the lavage fluid from the nitric acid exposed groups ($p < .10$) (Figure 5a). There was no increase in lavage fluid protein content in the group exposed to ozone alone, or ozone plus nitric acid. The elastase inhibitory capacity of lavage fluid from the group exposed to nitric acid alone was significantly increased (Figure 5b). There was no change in the elastase inhibitory activity of lavage fluid from the group exposed to ozone plus nitric acid or to ozone alone.

DISCUSSION

One purpose of this study was to examine a variety of macrophage-related endpoints

to determine their suitability for detecting the effects of atmospheres containing nitric acid. At least three types of endpoints were found to be affected by short term nitric acid exposure; 1) respiratory burst activity; 2) arachidonic acid metabolism; and 3) lavage fluid elastase inhibitory capacity. The relevance of these endpoints to human disease, and applicability of these endpoints to future studies of chronic episodic nitric acid exposure, is described below.

Respiratory Burst Activity

The term "respiratory burst" refers to the ability of phagocytes to generate a burst of superoxide in response to a variety of stimuli, including infectious agents. The importance of the respiratory burst in the immune defense system is dramatically demonstrated by the hereditary condition known as chronic granulomatous disease. Phagocytic cells from individuals with this disease cannot generate a respiratory burst, although the phagocytic capacity and other aspects of immune function are normal. This defect causes increased susceptibility to infection, and chronic micro-abscesses of the lung and liver--resulting in death in childhood or young adulthood (Ezekowitz et al., 1988).

A single 4 hour in vivo exposure to 1 mg/m^3 HNO_3 plus 0.6 ppm O_3 significantly decreased the respiratory burst activity (measured by lucigenin chemiluminescence) of lung lavage cells recovered 18 hours after exposure. Respiratory burst activity was unchanged (relative to controls) in lavage cells from animals exposed to nitric acid alone or to ozone alone. It is important to note that in this case, respiratory burst activity was measured after the lung lavage cells had been cultured for 20 hours to eliminate spontaneous respiratory burst activity. Macrophages in culture

spontaneously release a wide variety of factors, such as arachidonic acid metabolites, interferons and other cytokines which are known to modulate respiratory burst activity (Warren et al., 1988). Thus, the inhibitory effect of high levels of ozone and nitric acid on respiratory burst activity could be due to a direct effect on the respiratory burst mechanism or indirect effect on respiratory burst modulating factors.

In an attempt to clarify this issue, the respiratory burst activity was measured on freshly isolated cells and on cultured lavage cells. Exposure to 0.25 mg/m^3 HNO_3, 0.15 ppm O_3, or 0.25 mg/m^3 HNO_3 + 0.15 ppm O_3 4 hrs per day for 4 days had no effect on respiratory burst activity measured after 18 hours in culture. However, when respiratory burst activity was measured immediately after cell isolation, lung lavage cells from animals exposed to 0.25 mg/m^3 HNO_3 had significantly decreased respiratory burst activity. This suggests that repeated exposure to nitric acid may impair respiratory burst activity. Interestingly, increased rates of respiratory tract infection were noted in individuals living near a TNT plant who were exposed to high levels of NO_2 and presumably HNO_3 (Pearlman et al., 1971; Love et al., 1982). Thus, respiratory burst activity of lung lavage cells is a parameter that should be examined in future studies of chronic episodic nitric acid exposure.

Arachidonic Acid Metabolism

The arachidonic acid metabolites, leukotriene B_4 and leukotriene C_4, were selected for study because leukotriene B_4 is a potent neutrophil chemotactic agent and leukotriene C_4 (along with leukotriene D_4) is one of the slow reacting anaphyalaxins implicated in bronchospasm. Production of leukotriene B_4 in vitro was significantly elevated in lavage cells from rats after a single exposure to a high concentration of nitric acid.

It is not currently known how well in vitro arachidonic acid metabolism by lung macrophages reflects in vivo behavior. It should also be kept in mind that other cell types in the lung produce arachidonic acid metabolites and that lavage cells are only a very small fraction of the lung cell population. A much more informative picture of the effect of nitric acid exposure on pulmonary arachidonic acid metabolism can be obtained in future studies by correlating pulmonary function changes with arachidonic acid metabolite levels in lung lavage fluid and in macrophage conditioned medium. The present study has served to indicate that the above in-depth examination of arachidonic acid metabolism is warranted in future studies of nitric acid exposure.

During the course of these studies, we have become aware that the rat is a particularly good species for studying lung lavage cell respiratory burst activity and arachidonic acid metabolism because of the similarities between the behavior of human alveolar macrophages and rat alveolar macrophages. Alveolar macrophages from humans and from rats have high levels of spontaneous respiratory burst activity and high levels of arachidonic acid metabolism (Holtzman, 1991) when they are allowed to adhere to a culture dish (Hsueh et al., 1987; Williams and Cole, 1981a). In contrast, alveolar macrophages from rabbits show relatively little spontaneous respiratory burst activity or arachidonic acid metabolism during adherence (Hsueh et al., 1987). The biological significance of these species differences is not known; however, these differences must be kept in mind in interpreting results of future studies involving multiple species.

Lavage Fluid Elastase Inhibitory Capacity

We initially sought to examine the effect of nitric acid exposure on lung lavage cell elastolytic activity to determine if nitric acid exposure has the potential to cause

connective tissue damage. However, the lung lavage cells from all groups had little, if any, elastolytic activity and lung lavage fluid elastase-like activity was the same in all groups. We then measured elastase-inhibitory activity in lung lavage fluid. To our surprise we found that elastase inhibitory capacity was increased in lung lavage fluid from rats exposed to nitric acid, at both concentration levels (1 mg/m^3 for 1 day and 0.25 mg/m^3 for 4 days). The most likely explanation for this finding is that nitric acid exposure may have stimulated mucus secretion. While mucus is not thought of as an elastase inhibitor, it can probably act as a substrate for elastase, and thus competes with elastin for elastase in the assay mixture. Identification of the factors responsible for increased elastase inhibitory activity in the lavage fluid of acid exposed animals is clearly needed to interpret how this result relates to human health risks.

The most significant finding of this study was that (with one exception) the effects of ozone and nitric exposure were not additive. Not only did the two agents fail to show additive effects, but also in most cases the effect of ozone and nitric acid in combination was less than either ozone alone or nitric acid alone. For example, exposure to 0.6 ppm O_3 for 4 hours resulted in a two-fold increase in the lavage fluid protein content. Exposure to 1 mg/m^3 HNO_3 had no effect on protein content, so one might expect that exposure to both ozone and HNO_3 would have the same effect on lavage fluid protein content as ozone alone. However, animals exposed to both ozone and nitric acid had only a 1.5 fold increase ($p < .10$ ozone vs. ozone plus acid) in lung lavage protein. Another example is that exposure to 0.25 mg/m^3 HNO_3 for 4 hrs per day, 4 days a week, resulted in a significant increase in the lavage fluid elastase inhibitory capacity; however, no increase was seen in animals exposed to 0.15 ppm ozone plus 0.25 mg/m^3 HNO_3. Seemingly antagonistic effects of ozone and nitric acid

were also seen in endpoints relating to lavage cell number and cell type, leukotriene production and respiratory burst activity of freshly isolated lavage cells.

It has recently been reported (Aris et al., 1991) that prior exposure of humans to either nitric acid fog or water fog tended to attenuate ozone-induced changes in pulmonary function. While it is encouraging that our and Aris' studies do not indicate an additive interaction between ozone and nitric acid exposure in either rats or humans, it is important to realize that short-term acute studies on healthy individuals do not necessarily predict the effects of chronic repeated exposure of a diverse population. In addition, the ratio of nitric acid to ozone was much higher in the present study than is known to occur in ambient conditions.

In summary, this pilot study has identified three parameters which show promise of being sensitive indicators of nitric acid exposure. These parameters are 1) production of arachidonic acid metabolites by macrophages, 2) respiratory burst activity of lung macrophages, and 3) lavage fluid elastase inhibitory capacity. Respiratory burst activity and lavage fluid elastase inhibitory capacity are potentially useful for examining the effects of subchronic or chronic exposure to nitric acid. Results of this study do not indicate a synergistic interaction between ozone and nitric acid, in fact for some of the endpoints examined the effects of ozone and nitric acid were less than additive.

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PUBLICATIONS AND PRESENTATIONS

Publications

1. Nadziejko C.E., Wu S. Mechanism of lucigenin chemiluminescence of freshly isolated alveolar macrophages. J. Cell Biol. 1990; 111:309a.

2. Nadziejko C.E., Nansen L., Mannix R., Kleinman M.T. Acute effects of nitric acid vapor and ozone on lung macrophage function. Amer. Rev. Resp. Dis. (abstract) in press.

Presentations

"Acute effects of nitric acid vapor on lung macrophage function" presented by C. Nadziejko at the "California Conference on Air Pollution", UC Riverside 9/11/90.

"Effects of air pollution on health" presented by R. Phalen at the "Future of Air Quality and Transportation in the South Coast Basin" Conference, Raincross Square, Riverside, 10/30/91.

"Controlled animal studies" presented by R. Phalen at the "Workshop on Health Effects of Respirable Particulate Pollution", Brigham Young University, Salt Lake City, UT, 3/19/92.

REPORT DOCUMENTATION PAGE

1. AGENCY USE ONLY *(Leave Blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
PB94218880	February 1992	Final Report

4. TITLE AND SUBTITLE

Effects of Acidic Mixtures on Pulmonary Macrophage Functions: A Pilot Study

5. FUNDING NUMBERS

A933-078

6. AUTHOR(S)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

California Air Resources Board
Research Division
2020 L Street
Sacramento, CA 95814

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

ARB/R-94/541

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Release unlimited. Available from National Technical Information Service.
5285 Port Royal Road
Springfield, VA 22161

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Fischer 344 rats were examined for effects of inhaled nitric acid and ozone on macrophage cell function, to evaluate new endpoints for future acid inhalation studies. Pulmonary macrophage respiratory burst activity, production of arachidonic acid metabolites (leukotriene B4 and leukotriene C4) by macrophages, and lavage fluid elastase inhibitory capacity were found to be affected by *in vivo* exposure to nitric acid vapor, alone or in combination with ozone. These results have implications with respect to the development of lung infections, asthma, and emphysema. Rats exposed to 1 mg/m3 HNO3 for 4 hours once, and rats exposed to 0.25 mg/m3 HNO3 for 4hrs/day for 4 days had comparable increase in lavage fluid elastanse inhibitory capacity relative to controls exposed to air, suggesting that this endpoint may reflect the effects of nitric acid exposure.

14. SUBJECT TERMS

air pollution, human health, nitric acid vapor, pulmonary macrophage functions, new endpoints for future studies, acid inhalation

15. NUMBER OF PAGES

49

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited